



Multi-Organ Histopathology and Systemic Safety Profile of a High-Dose Recombinant Nipah Vaccine Candidate Formulated with SWE Adjuvant in Syrian Hamsters (*Mesocricetus auratus*)

ZAKIYYAN I. AYYUBA^{1,2}, REVIANY V. NIDOM^{2,3,4*}, SETYARINA INDRASARI^{2,3}, ASTRIA N. NIDOM^{2,5}, ELISA D. PRATIWI^{1,2}, TIZA W. MAWADDAH^{1,2}, IRMA Y. ROSITANIA^{1,2}, ESSA AL-RASSAS^{1,2}, RIFKY GAENAYAE², KUNCORO P. SANTOSO¹, KADEK RACHMAWATI¹, DJOKO LEGOWO¹, JOLA RAHMAHANI¹, LITA R. YUSTINASARI¹, CHAIRUL A. NIDOM^{1,2,3*}

¹Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia; ²Professor Nidom Foundation, Surabaya, Indonesia; ³Riset AIRC Indonesia, Surabaya, Indonesia; ⁴Department of Pharmacy, Faculty of Health Science, Universitas PGRI Adi Buana Surabaya; ⁵Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.

Abstract | Nipah virus (NiV) causes severe zoonotic disease with high fatality rates. Preclinical toxicity screening of recombinant attachment glycoprotein (G) seed vaccine candidates under repeated high-dose exposure is essential to confirm host tolerance limits. This study evaluated the repeated-dose systemic toxicity and multi-organ histopathology safety profile of a high-dose recombinant NiV seed vaccine candidate formulated with Squalene Oil-in-Water Emulsion (SWE) adjuvant in Syrian hamster (*Mesocricetus auratus*), using the Patterned Physiological Criteria framework evaluates synchronized multi-organ clearance responses against non-target tissue structural preservation. Twenty-four specific-pathogen-free Syrian hamsters were randomly allocated into four experimental groups (n=6/group, sex-balanced): P1 received high-dose vaccine antigen (200 μ g/mL, 0.05 mL); P2 received high-dose vaccine formulated with SWE adjuvant (1:1 v/v); P3 received distilled water; and P4 received SWE placebo. Formulations were administered via intramuscular injection at weeks 0 and 4. Daily clinical monitoring and body weights were maintained until week 8, whereupon total necropsy and semi-quantitative INHAND microscopic organ scoring (Grades 0–4) were executed. Inter-organ score relationship were evaluated using Spearman's rank correlation. Zero mortality (0/24) and no clinical distress were recorded across all cohorts over 56-day. Gross necropsy revealed normal visceral integrity without hemorrhages or gross structural abnormalities. Microscopic evaluation showed that repeated high-dose exposure induced only mild cellular adaptations (Grades ≤ 2) in the liver, lungs, and kidneys, as well as splenic white pulp hyperplasia (Grade 1). Non-target vital systems (heart, brain, intestine, and gonads) exhibited Grade 0 across all animals. Inter-organ analysis showed strong synchronized positive linear correlations ($p < 0.01$), anchored by the lung-spleen axis ($r_s = 0.944$) and liver-lung axis ($r_s = 0.919$). Recombinant NiV seed vaccine candidate formulated with SWE adjuvant demonstrated a favorable systemic safety profile in Syrian hamsters. Microscopic variations were interpreted as potential adaptive cellular responses during macromolecular processing. Further preclinical evaluation of immunogenicity and protective efficacy is recommended.

Keywords | Recombinant vaccine, Preclinical safety, Syrian hamster, Squalene emulsion, Histopathology, Experimental cohort, Biochemical indices

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*Correspondence | Reviany V. Nidom, Chairul A. Nidom, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia; ²Professor Nidom Foundation, Surabaya, Indonesia; Riset AIRC Indonesia, Surabaya, Indonesia; Department of Pharmacy, Faculty of Health Science, Universitas PGRI Adi Buana Surabaya; Email: nidomca@fkh.unair.ac.id or reviany@pnfinstitute.org

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INTRODUCTION

Nipah virus (NiV) represents a highly lethal zoonotic *Henipavirus* that poses a severe threat to global health security through recurring spillovers events and pandemic potential (Klingelhöfer *et al.*, 2025; Madhukalya *et al.*, 2025). Viral attachment glycoprotein (G) binding to host ephrin-B2/B3 receptors drives widespread microvascular disruption, microthrombosis, and hyperinflammatory host responses, precipitating fatal acute respiratory distress syndrome (ARDS) and necrotizing encephalitis (Brown *et al.*, 2023; Al-Obaidi *et al.*, 2024; Satapathy *et al.*, 2025). Among emerging vaccine technologies, recombinant subunit platforms targeting the attachment (G) glycoprotein offer key advantages over mRNA and viral vector platforms, including an absence of pre-existing anti-vector immunity, high thermal stability, an established non-replicating safety profile, and consistent industrial-scale manufacturing scalability (Li *et al.*, 2020; Gao *et al.*, 2022; Watanabe *et al.*, 2023; Mishra *et al.*, 2024). Industrial-scale manufacturing mandates rigorous safety screening of the master vaccine seed lot under repeated worst-case high-dose scenarios (200 µg/mL) to define biological tolerance thresholds prior to human clinical trials (Sasaki *et al.*, 2018; Sakurai *et al.*, 2019; Soleimani, 2022).

Formulating non-replicating antigens with advanced Squalene Oil-in-Water Emulsion (SWE) adjuvants enhances immune longevity, yet systemic biodistribution of submicron oil droplets introduces potential risks of transient reactogenicity, acute-phase hepatic clearance burdens, and transient microvascular neutrophil margination in pulmonary capillary beds (Ott *et al.*, 2000; Calabro *et al.*, 2011; Facciola *et al.*, 2022; Zhao *et al.*, 2023). Evaluating these biological responses requires an appropriate animal model, with Syrian hamsters (*Mesocricetus auratus*) serve as a translational model of choice due to their microvascular endothelial sensitivity and ephrin-B2/B3 receptor distribution mirroring human tissue targets, allowing safety to be established in the primary small animal species used

for Henipavirus efficacy testing (Wong and Ong, 2011; Miao *et al.*, 2019; Pigeaud *et al.*, 2023; Lv *et al.*, 2025).

Nonclinical safety guidelines (ICH S6, WHO guidelines) and standard diagnostic frameworks such as International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) mandate multi-organ histopathology evaluation (Greaves, 2012; Meyerholz and Beck, 2020). However, traditional interpretive approaches often evaluate organ pathology as isolated endpoints, creating ambiguity when distinguishing expected, coordinated clearance responses from uncoupled systemic toxicity (Bossart *et al.*, 2009; Geisbert *et al.*, 2010). To address this interpretive ambiguity, the “Patterned Physiological Criteria” as an exploratory analytical framework designed to complement established guidelines by mapping co-directional organ clearance trajectories. Operationally, this proposed exploratory framework defines host safety through two quantitative criteria: (1) statistically significant positive rank correlations exceeding an exploratory threshold of $r_s > 0.70$ representing strong positive rank concordance based on established statistical conventions (Mukaka, 2012; Akoglu, 2018) among responsive clearance organs (liver, lungs, kidneys, spleen) exhibiting low-grade, reversible cellular adaptations ($\leq$ Grade 2), and (2) unvaried Grade 0 structural preservation in non-target vital systems (brain, heart, intestine, gonads). This study evaluated the repeated-dose systemic toxicity and multi-organ histopathology safety profile of a high-dose SWE-adjuvanted recombinant NiV seed vaccine candidate in juvenile Syrian hamsters under this exploratory framework, establishing a safety baseline for further preclinical development.

MATERIALS AND METHODS

ETHICAL STATEMENT

All experimental procedures involving animals were conducted in strict accordance with the national guidelines for animal care and use. Institutional ethical approval was granted by the Animal Care and Use Committee

(IACUC) under approval number Approval No. 030623/IACUC/VII/2023. In vivo experimental procedures, immunization protocols, daily clinical monitoring, and necropsy operations were executed within the certified animal Biosafety Level 3 (BSL-3) facility at Professor Nidom Foundation (PNF), Surabaya, Indonesia.

Hamsters were housed in standard polycarbonate cages (42×27×18" cm") lined with autoclaved paper bedding and equipped with wooden chew blocks for environmental enrichment. Animals were housed 3 per cage (segregated by sex) under controlled environmental conditions (temperature 22±3° "C", relative humidity 55±10%, 12-hour light/dark cycle) with a standard commercial pellet diet and water provided *ad libitum*. Tissue processing, paraffin embedding, microtomy, Hematoxylin-Eosin (HE) staining, digital micro-imaging, and semi-quantitative INHAND microscopic scoring were performed at the Animal Diagnostic Laboratory (PADIA-Lab), PNF, Surabaya, Indonesia.

EXPERIMENTAL DESIGN

Twenty-four specificpathogen-free (SPF) juvenile Syrian hamsters (*Mesocricetus auratus*), aged 3–4 weeks with body weights ranging between 20–30 grams were obtained from certified breeding stocks. Sample size calculation was determined using Mead's Resource Equation (Mead, 1990) to adhere to the Reduction principle of the 3Rs framework:

$$E=N-T$$

Where N represents the total number of experimental units (N=24) and T represents the total degrees of freedom for the treatment factor combinations. Accounting for a 4×2 factorial design (4 treatment conditions × 2 sexes; T=8), the error degrees of freedom is:

$$E=N-T=24-8=16$$

An E value of 16 satisfies the ideal regulatory threshold (10≤E≤20), validating statistical power without animal redundancy. Animals were randomly assigned into four treatment groups n=6/group (3 males and 3 females): **P1 (High-Dose Vaccine)**: Received 0,05 mL intramuscular inoculation of a high-dose (200 μg/mL) recombinant Nipah virus attachment G-protein seed preparation; **P2 (High-Dose Vaccine + SWE Adjuvant)**: Received 0,05 mL intramuscular inoculation of the high-dose vaccine antigen (200 μg/mL) formulated with Squalene Oil-in-Water Emulsion (SWE) adjuvant at a 1:1 volume ratio; **P3 (Negative Control)**: Received 0,05 mL intramuscular injection of sterile distilled water; **P4 (Adjuvant Control)**: Received 0,05 mL intramuscular injection of placebo

SWE vehicle, composed of a squalene-in-water emulsion (squalene, polysorbate 80, and sorbitan trioleate in a isotonic citrate buffer system) formulated identically to the adjuvant component of P2 but without viral antigen

All test formulations were administered by intramuscular injection into the cranial thigh muscle (*musculus quadriceps femoris*), an injection volume of 0,05 mL per site avoids muscle tearing, local tissue tension, and formulation leakage, while injection into the quadriceps femoris eliminates the risk of sciatic nerve damage associated with posterior thigh routes. However, localized mechanical tissue tension induced by this volume in 20–30 g juvenile muscle mass is acknowledged as a potential minor confounding factor independent of vaccine reactogenicity. Prime immunization was administered on Day 0, followed by a booster immunization on Day 28, simulating a worst-case systemic exposure scenario. To optimize animal usage in accordance with the Reduction principle (3Rs) during this initial safety screening, a post-treatment recovery cohort was excluded. The study design focused resources on characterizing peak tissue reactogenicity at Day 56 (28 days post-booster) prior to committing to full-scale recovery evaluations.

HUSBANDRY AND CLINICAL OBSERVATION

Hamsters were acclimatized for 7 days under controlled temperature (22±3° "C"), relative humidity (55±10%), and a 12-hour light/dark cycle. Standard commercial pellet diet and fresh water were provided *ad libitum*. Animals were observed daily at 09.00 AM by blinded observers for qualitative clinical signs of distress or systemic toxicity, including lethargy, piloerection, respiratory distress, motor deficits, and injection site reactogenicity throughout the 56-day observation period. Body weight was recorded at baseline and prior to terminal necropsy at Week 8 to evaluate overall health maintenance and screen for treatment-related weight depression.

On Day 56, animals were euthanized following deep intramuscular anesthesia using a combination of ketamine (80" mg/kg body weight) and xylazine (5" mg/kg" body weight), followed by cervical dislocation. Full gross necropsy was performed immediately to inspect visceral organs for hemorrhages, lesions, or gross anatomical changes . Target organs (liver, lungs, kidneys, spleen, heart, brain, intestine, and gonads) were harvested and immersion-fixed in 10% Neutral Buffered Formalin (NBF) for 48 hours to ensure thorough tissue penetration prior to paraffin embedding.

TISSUE PROCESSING AND HISTOPATHOLOGY

Fixed tissue specimens were processed using an automated tissue processor. Dehydration was performed through ascending grade ethanol steps (70%, 80%, 95%, and 100%

for 1 hour each). Clearing was achieved by immersing tissues in xylene across three separate jars for 1 hour each. Impregnation was completed using molten pure paraffin wax across three separate containers for 1 hour each.

Processed tissues were embedded into paraffin blocks using a standard embedding center. Thin sectioning was performed using a Leica RM2125RTS microtome to obtain tissue ribbons with a thickness of 4-5 μm . Hematoxylin-Eosin (HE) staining was initiated with deparaffinization in xylene for three stages (5 minutes each). Rehydration followed via descending ethanol grades (100%, 95%, 80%, and 70% for 2 minutes each) before rinsing in running tap water for 5 minutes. Cell nuclei were stained with Mayer's hematoxylin for 10 minutes, rinsed in running tap water for 5 minutes, and cytoplasm was counterstained using 1% alcoholic eosin Y for 2 minutes. Final dehydration used 95% and 100% ethanol (1 minute each) followed by final clearing in xylene (5 minutes) (Greaves, 2012; Meyerholz and Beck, 2020). Section mounting was completed using glass cover slips and a xylene-based liquid mounting medium.

Microscopic evaluation was conducted by blinded pathologists using a Nikon® Eclipse Ci microscope integrated with digital software. Diagnostic nomenclature and lesion identification were standardized using INHAND criteria, tissue alterations were scored using an adapted semi-quantitative 5-point grading scheme (Grades 0–4) (Gibson-Corley *et al.*, 2013). Scoring prioritized primary morphological changes per organ evaluated by percentage tissue area involvement. The consolidated multi-organ histopathology scoring matrix is detailed in Table 1.

STATISTICAL ANALYSIS

Statistical computations were executed using IBM SPSS Statistics (Version 28.0). Data distributional traits were tested using the Shapiro-Wilk test, while variance homogeneity was assessed via Levene's test. Because histopathology data consist of ordinal semi-quantitative grades (Grades 0–4) exhibiting non-normal distributions ($p < 0.05$), non-parametric inferential methods were applied.

To evaluate potential sex-related differences, the Scheirer-Ray-Hare extensions of the Kruskal-Wallis test was conducted, which confirmed no significant main effects of Sex ($p > 0.05$) and no Treatment $\times$ Sex interactions ($p > 0.05$) across any organ system. Main treatment effects across experimental cohorts ($n = 6/\text{group}$) were evaluated using the Kruskal-Wallis test ($df = 3, \alpha = 0.05$). Post-hoc pairwise comparisons between treatment groups were conducted using the Dunn-Bonferroni test, with significance levels adjusted for the 6 pairwise comparisons within each organ family ($\alpha = 0.05/6 = 0.0083$).

To evaluate systemic response trajectories across the complete experimental exposure spectrum (from unexposed control to high-dose exposure), Spearman's rank correlation coefficient (r_s) were computed across the combined cohort ($n = 24$). Organs exhibiting unvaried Grade 0 across all cohorts were documented descriptively to confirm structural safety. Analytical roles of each statistical test are summarized in Table 2.

RESULTS

CLINICAL SAFETY AND NECROPSY

Intramuscular administration of the high-dose recombinant Nipah seed vaccine alone (P1) or formulated with SWE adjuvant (P2) produced zero mortality (0/24) across all experimental cohorts throughout the 56-day trial. Daily clinical monitoring confirmed normal behavioral patterns without signs of systemic distress, lethargy, piloerection, respiratory abnormalities, or motor deficits. Injected sites exhibited no swelling, erythema, or local reactivity.

All experimental cohorts exhibited steady, age-appropriate body weight gain over the 56-day observation period. Mean initial body weights ranged from 24.1 ± 2.0 g to 24.8 ± 1.9 g, and final body weights ranged from 110.1 ± 8.2 g to 114.3 ± 6.9 g. Systematic post-mortem examination revealed normal gross morphology across all visceral organs, with no evidence of congestion, petechial hemorrhage, or gross structural abnormalities. Gross necropsy findings are summarized in Table 3.

HISTOPATHOLOGY OF ACTIVE ORGANS

Semi-quantitative microscopic scoring revealed statistically significant treatment effects ($p < 0.05$) across the liver, lungs, kidneys, and spleen. Non-parametric Scheirer-Ray-Hare analysis confirmed no main effects of Sex ($p > 0.05$) and no Treatment $\times$ Sex interactions ($p > 0.05$). Consolidated median scores and post-hoc significance letterings across combined cohorts ($n = 6/\text{group}$) are summarized in Table 4.

Microscopic evaluation of liver tissue from control hamsters (P3 and P4) verified intact lobular architecture and normal hepatic cords (Figure 1A, B). Active treatment groups (P1 and P2) exhibited centrilobular focal vacuolar degeneration of hepatocyte (Grade 1–2) without evidence of ballooning necrosis or lobular structural disruption (Figure 1C–F). These low-grade changes are morphologically characteristic of reversible cellular adaptation during macromolecular processing.

Pulmonary sections from control cohorts presented thin interalveolar septa and patent alveolar spaces (Figure 2A, B). Inoculated animals (P1 and P2) showed mild interalveolar

Table 1: Consolidated scoring matrix for multi-organ histopathology evaluation.

Organ Target	Primary Diagnostic Lesion Evaluated	Score Definition and Tissue Grading Criteria (Grade 0–4)	Reference
Liver	Hepatocyte hydropic vacuolation	0: Normal parenchyma; 1: Minimal focal hydropic vacuolation (<10% lobular area); 2: Mild multifocal hydropic vacuolation (10–25% area); 3: Moderate hydropic vacuolation (26–50% area); 4: Severe diffuse hydropic degeneration (>50% area).	Thoolen <i>et al.</i> (2010); Gibson-Corley <i>et al.</i> (2013)
Lung	Interalveolar septal thickening	0: Normal alveolar architecture; 1: Minimal focal septal thickening (<10% lung field); 2: Mild multifocal septal thickening (10–25% lung field); 3: Moderate multifocal septal thickening (26 – 50% lung field) with focal edema; 4: Severe pulmonary consolidation (>50% lung field).	Renne <i>et al.</i> (2009); Gibson-Corley <i>et al.</i> (2013)
Kidney	Proximal tubular epithelial hydropic swelling .	0: Normal nephron architecture; 1: Minimal focal tubular hydropic degeneration (<10% cortical tubules); 2: Mild tubular hydropic degeneration (10 – 25% cortical tubules); 3: Moderate tubular hydropic degeneration (26 – 50% cortical tubules), hyaline casts present ; 4: Severe tubular hydropic degeneration with necrosis (>50% cortical tubules).	Frazier <i>et al.</i> (2012); Gibson-Corley <i>et al.</i> (2013)
Spleen	Follicular hyperplasia	0: Normal lymphoid architecture; 1: Minimal active germinal center formation (<10% white pulp follicles); 2: Mild follicular expansion (10 – 25% white pulp follicles); 3: Moderate follicular expansion (26 – 50% white pulp follicles); 4: Severe diffuse follicular expansion (>50% white pulp follicles).	Elmore (2006); Gibson-Corley <i>et al.</i> (2013)
Heart	Myocardial cell alteration/inflammation .	0: Normal branching syncytium; 1: Minimal focal cellular alteration (<10% myocardium); 2: Mild multifocal cellular alteration (10 – 25% myocardium); 3: Moderate myocyte alteration (26 – 50% myocardium); 4: Severe diffuse myocarditis (>50% myocardium) with necrosis.	Berridge <i>et al.</i> (2016); Gibson-Corley <i>et al.</i> (2013)
Brain	Cerebral cortical neuropil alteration	0: Normal neuronal morphology; 1: Minimal focal gliosis (<10% area evaluated) ; 2: Mild multifocal gliosis (10 – 25% area evaluated); 3: Moderate neuronal alteration (26 – 50% area evaluated); 4: Severe diffuse neuronal alteration (>50% area evaluated) with neuronal necrosis, encephalopathy.	Bradley <i>et al.</i> (2020); Gibson-Corley <i>et al.</i> (2013)
Intestine	Mucosal villous leukocyte infiltration	0: Intact villi and normal mucosa; 1: Minimal villous leukocyte infiltration (<10% mucosal area); 2: Mild lamina propria infiltration (10 -25% mucosal area); 3: Moderate mucosal disruption (26 – 50% mucosal area); 4: Severe mucosal disruption (>50% mucosal area) including ulceration.	Erben <i>et al.</i> (2014); Gibson-Corley <i>et al.</i> (2013)
Testis	Seminiferous tubular vacuolation	0: Normal physiological maturation; 1: Minimal tubular vacuolation (<10% area evaluated); 2: Mild tubular vacuolation/alteration (10 – 25% area evaluated); 3: Moderate tubular degeneration (26 – 50% area evaluated); 4: Severe tubular alteration/atrophy (>50% area evaluated).	Creasy <i>et al.</i> (2012); Gibson-Corley <i>et al.</i> (2013)
Ovary	Follicular atresia	0: Normal physiological maturation; 1: Minimal follicular alteration, atresia (<10% area evaluated); 2: Mild follicular alteration, atresia (10 – 25% area evaluated); 3: Moderate follicular alteration, atresia (26 – 50% area evaluated); 4: Diffuse follicular loss, atresia (>50% area evaluated).	Dixon <i>et al.</i> (2014); Gibson-Corley <i>et al.</i> (2013)

Table 2: Summary of statistical tests and analytical roles.

Statistical Test Method	Specific Data Target	Analytical Role and Decision Threshold
Shapiro-Wilk Test	Histopathological scores (n=24)	Assesses distributional normality (p<0.05 indicates non-normal distribution guiding non-parametric test selection)
Levene's Test	Group score variances (n=6/"group")	Evaluates homogeneity of variance (p<0.05 indicates non-homogeneous variance)
Scheirer-Ray-Hare Test	Factorial scores (4" Treatments"×2" Sexes")	Evaluates main effects of Treatment, Sex, and Treatment × Sex interaction (α=0.05).
Kruskal-Wallis Test	Between-group score comparisons (df=3)	Computes overall treatment effect significance across cohorts (n=6/"group",p<0.05).
Dunn-Bonferroni Test	Post-hoc pairwise comparisons (6 pairs/organ)	Identifies specific group differences and assigns significance letters based on organ-wise adjusted p-values (α=0.0083).
Spearman's Rho (r _s)	Inter-organ score pairs (n=24)	Maps co-directional multi-organ clearance trajectories across the experimental exposure continuum (p<0.01)

Table 3: Necropsy and gross pathology checklist across experimental cohorts.

Evaluation Parameter	P1 (High-Dose Vaccine)	P2 (Vaccine + SWE)	P3 (Distilled Water)	P4 (SWE Placebo)
Mortality Rate	0%	0%	0%	0%
Clinical Distress Signs	Absent	Absent	Absent	Absent
Body Weight Progression	Normal	Normal	Normal	Normal
Injection Site Reactogenicity	None	None	None	None
Hepatic Gross Appearance	Normal size/color	Normal size/color	Normal size/color	Normal size/color
Pulmonary Gross Appearance	Fully inflated/pink	Fully inflated/pink	Fully inflated/pink	Fully inflated/pink
Renal Gross Appearance	Intact capsule	Intact capsule	Intact capsule	Intact capsule
Splenic Gross Appearance	Normal gross appearance	Normal gross appearance	Normal gross appearance	Normal gross appearance
Visceral Hemorrhages	Absent	Absent	Absent	Absent

Table 4: Histopathology median grades (min–max) of active organs.

Organ Target	Experimental Cohort	Male	Female	p-value
Liver	P1	1 (1-2) ^a	1 (1-2) ^a	p_ "Treatment" =[[0.00033]] [*] p_ "Sex" =0.60635 p_ "Interaction" =0.85029
	P2	1 (1-2) ^a	1 (0-1) ^{ab}	
	P3	0 (0-0) ^b	0 (0-0) ^b	
	P4	0 (0-0) ^b	0 (0-0) ^b	
Lung	P1	2 (1-2) ^a	2 (1-2) ^a	p_ "Treatment" =[[0.00013]] [*] p_ "Sex" =0.85033 p_ "Interaction" =0.99101
	P2	1 (1-2) ^a	2 (1-2) ^a	
	P3	0 (0-0) ^b	0 (0-0) ^b	
	P4	0 (0-0) ^b	0 (0-0) ^b	
Kidney	P1	1 (1-1) ^a	1 (1-2) ^a	p_ "Treatment" =[[0.00050]] [*] p_ "Sex" =0.86761 p_ "Interaction" =0.99376
	P2	1 (0-1) ^{ab}	1 (0-1) ^{ab}	
	P3	0 (0-0) ^b	0 (0-0) ^b	
	P4	0 (0-0) ^b	0 (0-0) ^b	
Spleen	P1	1 (1-1) ^a	1 (1-1) ^a	p_ "Treatment" =[[0.00004]] [*] p_ "Sex" =1.000 p_ "Interaction" =1.000
	P2	1 (1-1) ^a	1 (1-1) ^a	
	P3	0 (0-0) ^b	0 (0-0) ^b	
	P4	0 (0-0) ^b	0 (0-0) ^b	

Note: Values represent Median (Min–Max). Main treatment effects (p_ "Treatment"), sex main effects (p_ "Sex"), and interaction effects (p_ "Interaction") were calculated using the Scheirer-Ray-Hare extension of the Kruskal-Wallis test (α=0.05). p_ "Sex" =

1.000 and $p_{\text{Interaction}} = 1.000$ for splenic white pulp hyperplasia resulted mathematically from completely identical median score distributions between male and female cohorts across all treatment groups. Asterisks (*) indicate significant main treatment effects ($p < 0.05$). Different superscript letters (a,b,ab) within the same column for an individual organ indicate statistically significant differences determined by the Dunn-Bonferroni post-hoc test ($p < 0.05$).

Table 5: Spearman's rho correlation matrix of responsive organs.

Organ Target	Liver	Lung	Kidney	Spleen
Liver	--			
Lung	0.919**	--		
Kidney	0.749**	0.814**	--	
Spleen	0.886**	0.944**	0.834**	--

Note: Values represent Spearman's rank correlation coefficient (r_s). The superscript symbol ** indicates that the correlation is highly significant at the $p < 0.01$ level. Non-target vital organs exhibiting Grade 0 across all subjects (heart, brain, intestine, and gonads) possessed zero variance and evaluated descriptively.

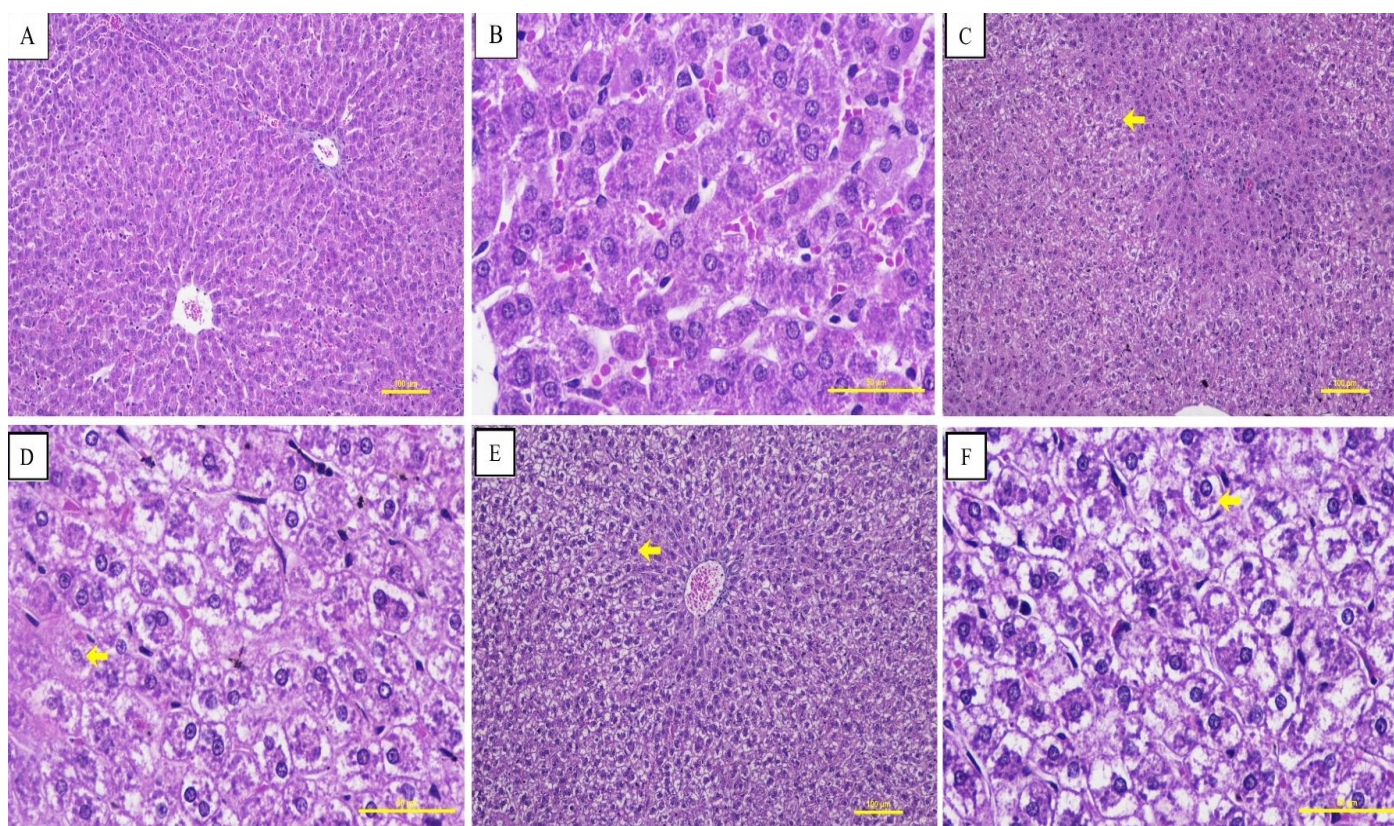


Figure 1: Microscopic features of Syrian hamster hepatic tissue (HE staining). (A) Grade 0 (100×) and (B) 400× magnification from group P3 showing normal lobular cords; (C) Grade 1 (100×) and (D) 400× magnification from P2 group; (E) Grades 2 (100×) and (F) 400× magnification from P1 group. Arrows indicate focal vacuolar degeneration (Scale bars: 100× = 200 μm; 400× = 50 μm).

septal thickening associated with focal neutrophilic PMN infiltration (Figure 2C–F), indicating localized acute inflammatory reactivity. Alveolar lumen patency was fully maintained across all fields without pulmonary edema or intra-alveolar hemorrhage.

Renal architecture in control animals presented intact glomeruli and cortical tubules (Figure 3A, B). Active cohorts (P1 and P2) exhibited focal vacuolar degeneration restricted to the proximal tubular epithelium (Figure 3C–F). Glomerular tufts, Bowman's spaces, and tubular basement membranes remained intact across all groups

without proteinaceous cast formation.

Splenic tissue from control hamsters showed resting lymphoid follicles (Figure 4A, B). Active treatment cohorts (P1 and P2) displayed a uniform shift to Grade 1, characterized by reactive white pulp follicular B-cell hyperplasia and germinal center expansion (Figure 4C, D). Expanded follicles were bounded by intact mantle zones without white pulp lymphoid depletion or red pulp congestion, reflecting reactivity following antigen/adjuvant administration.

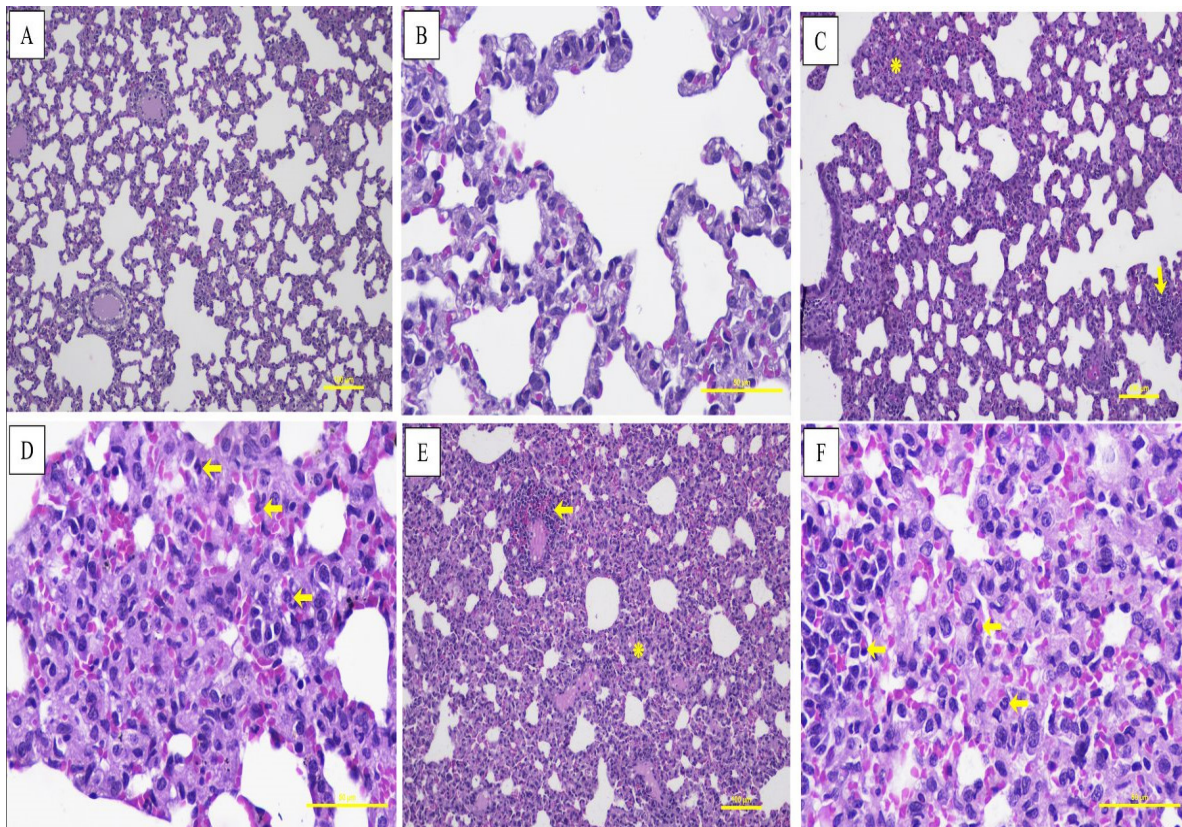


Figure 2: Microscopic features of Syrian hamster pulmonary tissue (HE staining). (A) Grade 0 (100×) and (B) 400× magnification from control group P4; (C) Grade 1 (100×) and (D) 400× magnification from P2 group; (E) Grades 2 (100×) and (F) 400× magnification from P1 group. Arrows denote PMN neutrophil infiltration; asterisks (*) mark interalveolar septal thickening (Scale bars: 100× = 200 μm; 400× = 50 μm).

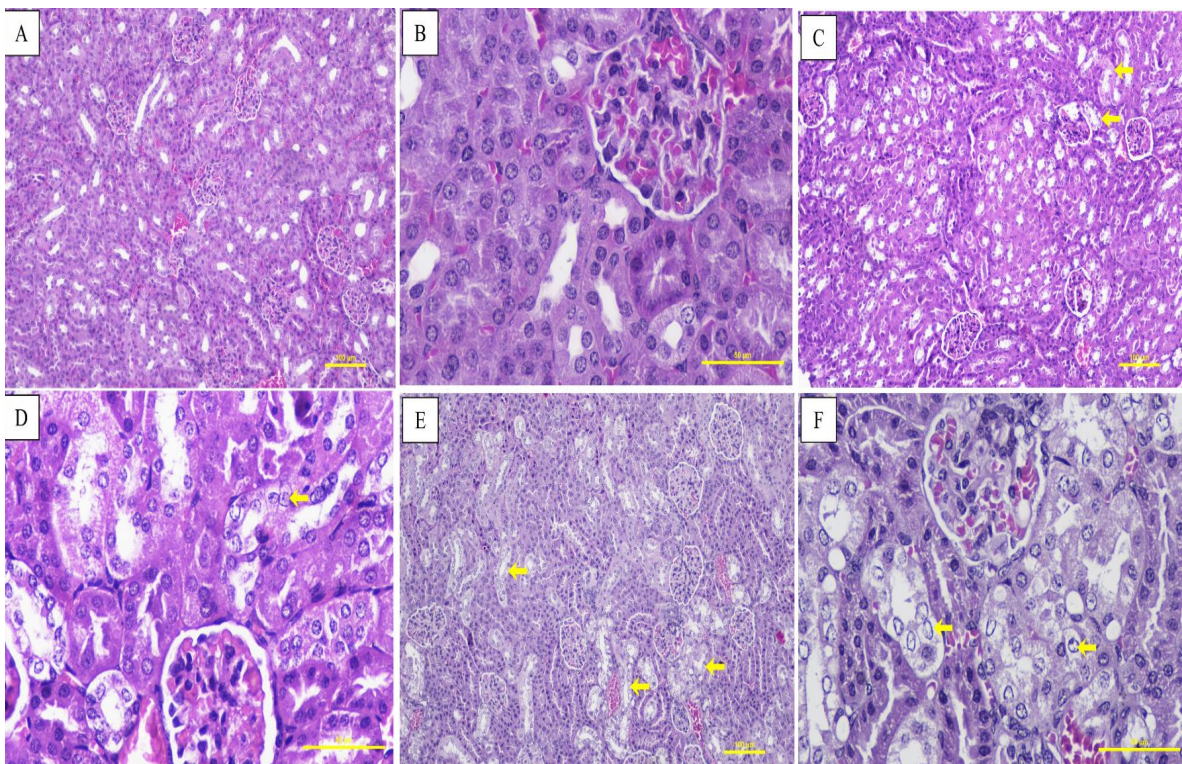


Figure 3: Microscopic features of Syrian hamster renal tissue (HE staining). (A) Grade 0 (100×) and (B) 400× magnification from control group P3; (C) Grade 1 (100×) and (D) 400× magnification from P2 group; (E) Grades 2 (100×) and (F) 400× magnification from P1 group. Arrows indicate proximal tubular epithelial vacuolar degeneration (Scale bars: 100× = 200 μm; 400× = 50 μm).

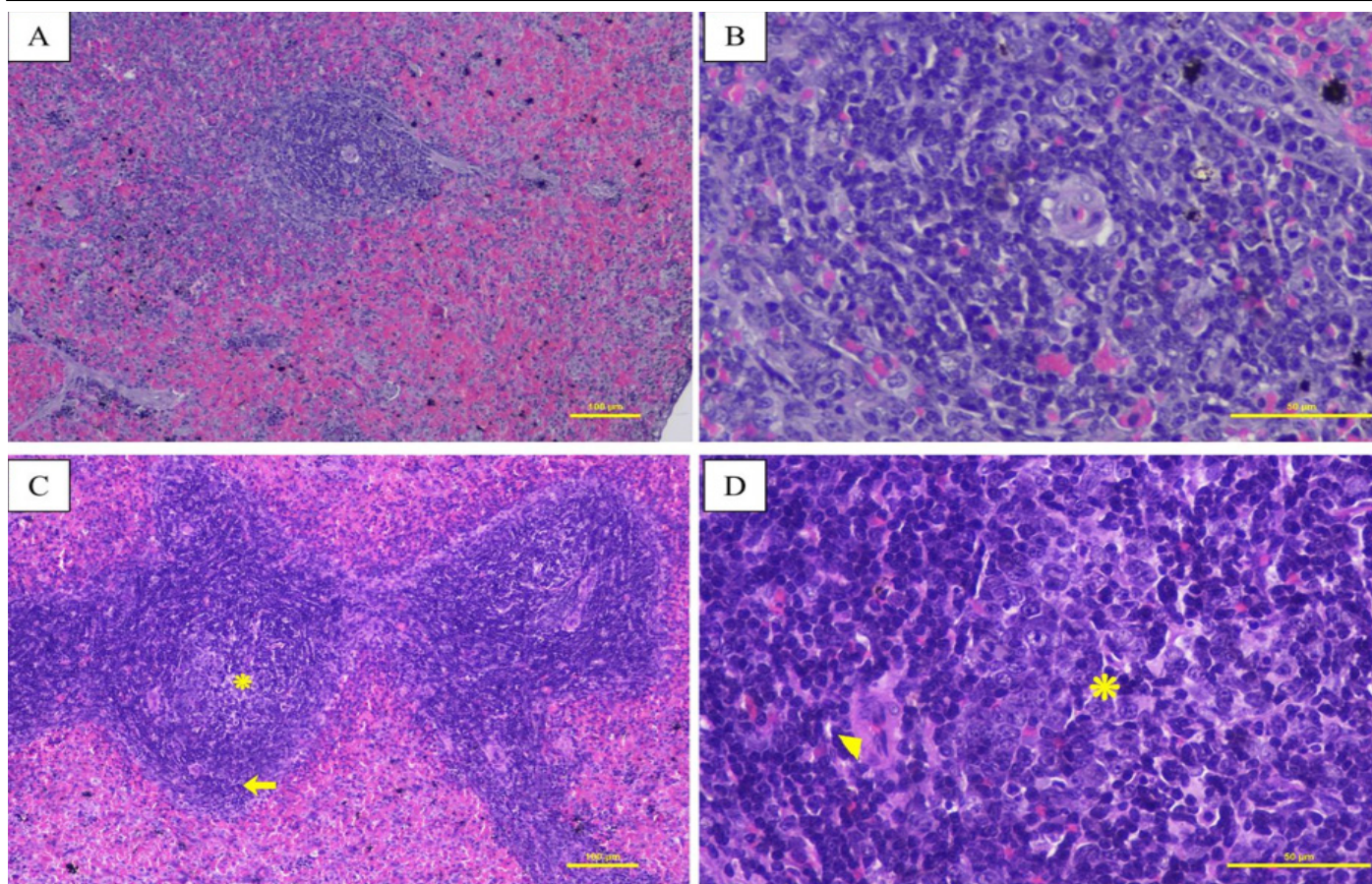


Figure 4: Microscopic features of Syrian hamster splenic tissue (HE staining). (A) Grade 0 (100×) and (B) 400× magnification from control group P4; (C) Grade 1 (100×) and (D) 400× magnification from P2 group. Arrows mark the intact mantle zone; asterisks (*) denote germinal center proliferation; arrowheads locate central arterioles (Scale bars: 100× = 200 μm; 400× = 50 μm).

HISTOPATHOLOGY OF NON-TARGET ORGANS

Microscopic evaluation of life-sustaining non-target organs (heart, brain, intestine, and gonads) demonstrated complete structural preservation. All subjects across all cohorts achieved unvaried Grade 0.

Myocardial tissue retained normal branching myofiber syncytia without necrosis, hyaline degeneration, or inflammatory cell infiltration (Figure 5A). Cerebral cortical neuropil maintained intact cellular architecture without evidence of microglial gliosis, neuronal necrosis, or perivascular cuffing (Figure 5B). Intestinal mucosal sections showed intact villi architecture and normal lamina propria without mucosal erosion or ulceration (Figure 5C). Gonadal tissues displayed physiological maturation evidenced by active spermatogenesis in seminiferous tubules (Figure 5D), and normal follicular development in ovaries (Figure 5E).

PATTERNED PHYSIOLOGICAL CRITERIA CORRELATION MATRIX

To evaluate systemic response trajectories across the experimental exposure spectrum (from unexposed baseline to high-dose exposure), Spearman's rank correlation

analysis was performed across all active responsive organs (n=24). Statistically significant positive rank correlations ($p < 0.01$) were recorded among all active organ pairs (Table 5).

The lung-spleen axis recorded the highest rank correlation coefficient ($r_s = 0.944$), followed closely by the liver-lung axis ($r_s = 0.919$), liver-spleen axis ($r_s = 0.886$), spleen-kidney axis ($r_s = 0.834$), lung-kidney axis ($r_s = 0.814$), and liver-kidney axis ($r_s = 0.749$). These global rank correlations reflect cohort-wide, treatment-induced score distributions driven primarily by group-level separation across exposure conditions (Grade 0 in unexposed controls versus Grade 1-2 in active cohorts), rather than demonstrating direct intra-individual causal mechanisms or synchronized homeostatic clearance.

DISCUSSION

Preclinical safety evaluation of biological master seed lots under repeated worst-case high-dose exposure scenarios is essential to define host tolerance limits prior to clinical trials (Sasaki *et al.*, 2018; Vervaeke *et al.*, 2022). In this study, intramuscular administration of a high-dose recombinant

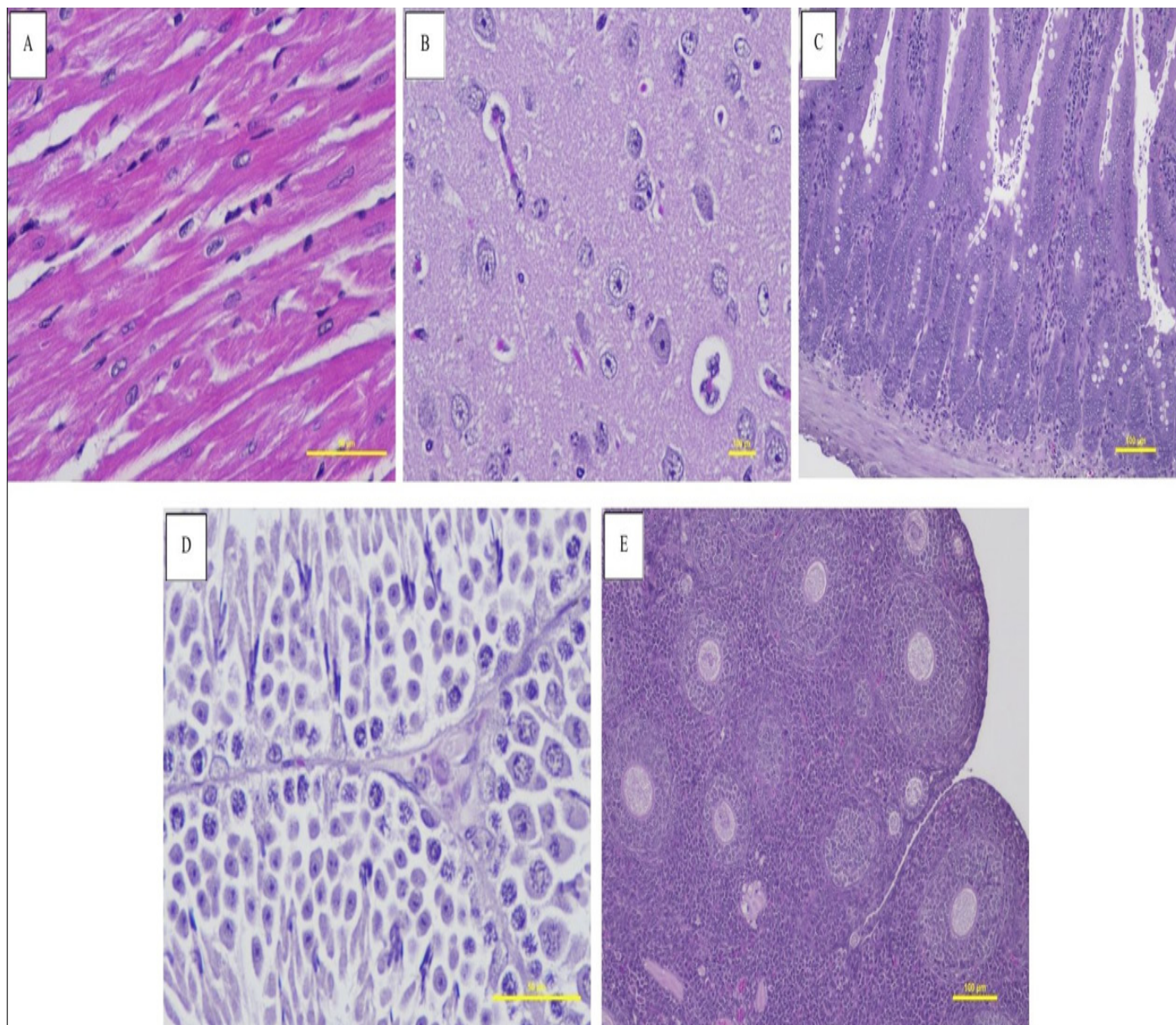


Figure 5: Representative microscopic panels of non-target vital organs demonstrating Grade 0 structural preservation. (A) Normal cardiac myocardium; (B) Intact cerebral cortex neuropil; (C) Normal intestinal mucosal villi; (D) Testicular seminiferous tubules showing active spermatogenesis; (E) Ovarian parenchyma demonstrating normal follicular maturation (Scale bars: 100 $\times$ = 200 μ m; 400 $\times$ = 50 μ m).

Nipah seed vaccine formulated with SWE adjuvant demonstrated high host tolerance in Syrian hamsters, contrasting with wild-type henipavirus infections that cause lethal microvascular disruption, fatal respiratory distress, and necrotizing encephalitis (Wong and Ong, 2011; Pigeaud *et al.*, 2023).

Microscopic examination showed mild, localized cellular changes in the liver, lungs, and kidneys (Grades ≤ 2). Hepatic cellular swelling changes may represent an adaptive metabolic response during macromolecular clearance (Thoolen *et al.*, 2010; Kumar *et al.*, 2020). Elevated metabolic demands on hepatocytes during high-dose antigen biotransformation provide a plausible explanation for transient intracellular

fluid accumulation without structural ballooning necrosis or lobular disruption (Cheville, 2009; Zachary and McGavin, 2017). Inter-alveolar septal thickening and focal PMN neutrophilic infiltration in lungs reflects localized acute inflammatory reactivity to circulating squalene microemulsion droplets (Calabro *et al.*, 2011; Klucker *et al.*, 2012). This localized inflammatory reactivity occurred without compromising alveolar gas exchange or inducing pulmonary edema (Slauson and Cooper, 2002; Cantor, 2025). Renal proximal tubular vacuolar degeneration represents an established physiological response during macromolecular filtration and tubular reabsorption (Frazier *et al.*, 2012; Kurts *et al.*, 2025), occurring without glomerular damage or proteinaceous cast formation (Jang

et al., 2020; Schnellmann, 2022).

Splenic white pulp follicular B-cell hyperplasia (Grade 1) is consistent with immune reactivity following administration of adjuvanted protein antigen (Seubert *et al.*, 2008). Squalene oil-in-water emulsions prolong antigen presentation, optimizing systemic immune engagement within regional lymphoid tissues (Ott *et al.*, 2000). Controlled germinal center expansion bounded by intact mantle zones signifies an organized reactive immune response rather than immunopathology (Seubert *et al.*, 2008).

Non-target vital systems (heart, brain, intestine, gonads) maintained Grade 0 across all cohorts, demonstrating host structural preservation at the tested dose and 56-day observation window. The absence of microglial gliosis or perivascular cuffing indicates that recombinant attachment glycoproteins (G) macromolecules did not alter cerebral neuropil architecture under physiological conditions (Daneman and Prat, 2015; Banks, 2016). Likewise, intact myocardial syncytia, intestinal mucosal villi, and gonadal architectures confirm structural tolerance across non-target tissues (Creasy *et al.*, 2012; Erben *et al.*, 2014; Berridge *et al.*, 2016; Li *et al.*, 2024; Gupta *et al.*, 2025).

Application of the proposed exploratory Patterned Physiological Criteria framework provided an integrative analytical approach to evaluate multi-organ host responses across exposure levels. While the strong inter-organ rank correlation ($r_s=0.749-0.944, p<0.01$) are driven primarily by group-level score separation between unexposed control cohorts and active treatment groups rather than establishing direct physiological causation, the concurrent manifestation of low-grade cellular adaptations across clearance tissues (liver, lungs, kidneys, spleen)—without isolated severe pathology in any single organ—is consistent with broad host tolerance across clearance pathways. Lung-spleen axis ($r_s=0.944$) aligns with established pulmonary-splenic immune axes, where splenic B-cell activation regulates endothelial cell adhesion and leukocyte recruitment within pulmonary capillaries (Didierlaurent *et al.*, 2014; Choque-Guevara *et al.*, 2022; Yang *et al.*, 2026). The liver-lung axis ($r_s=0.919$) demonstrates coordinated metabolic clearance between hepatic parenchymal cells and pulmonary intravascular macrophages (Cullen and Stalker, 2016; Pratiwi *et al.*, 2026). Complementary excretory balancing across liver-kidney ($r_s=0.749$), lung-kidney ($r_s=0.814$), and spleen-kidney ($r_s=0.834$). While the global correlations are driven primarily by treatment group assignments across the cohort, the concurrent low-grade alterations across filtration and immune organs—without isolated severe pathology in any single organ—support the conclusion that microscopic changes represent an organized systemic clearance process rather than

uncoupled, idiosyncratic toxicity (Giacomini *et al.*, 2010; Hur *et al.*, 2025).

This multi-organ evaluation directly satisfies regulatory requirements established by BPOM Regulation No. 10 of 2022, WHO guidelines for nonclinical evaluation of vaccines, US-FDA guidance on starting dose safety, and EMA vaccine standards. Specifically, the study meets key regulatory criteria by validating repeated worst-case dosing safety, 56-day post-immunization observation stability, sex-balanced cohort tolerance, and standardized INHAND multi-organ histopathology screening (WHO, 2005; FDA, 2005; EMA, 2010; BPOM, 2022).

STUDY LIMITATIONS AND RECOMMENDATION

Several methodological constraints warrant consideration when interpreting these safety findings, including the absence of a post-treatment recovery cohort to verify lesion reversibility, serial euthanasia time-points to characterize acute reactivity kinetics, dose-ranging groups to establish NOAEL, immunogenicity correlates (neutralizing antibody titers, IgG subclasses, cellular responses), protective challenge data, comparative adjuvant arms, injection site histopathology, quantitative organ weights, longitudinal body weight trajectories, biochemical/hematological parameters, and non-human primate validation, while the exclusive use of juvenile hamsters (3–4 weeks old) with limited group sizes ($n=6/\text{group}$, $n=3/\text{sex}$) constrains extrapolation to adult populations and statistical power for detecting subtle sex-specific responses.

Future investigations should incorporate dose-ranging toxicity studies with dedicated recovery cohorts to establish NOAEL and lesion reversibility kinetics, execute serial time-point harvests to characterize the temporal evolution of tissue responses, expand cohort sizes to $n=10-12/\text{group}$ to enhance statistical power for sex-specific analyses, perform detailed histopathological evaluation of local injection sites with quantitative organ weight assessments, conduct integrated immunogenicity and protective efficacy studies in both juvenile and adult hamster models, benchmark SWE adjuvant against established adjuvants (alum, MF59), and ultimately validate systemic safety and protective efficacy in non-human primate models prior to human clinical trial progression, with concurrent serum biochemistry and hematological monitoring to complement histopathological evaluations.

CONCLUSION

Repeated-dose intramuscular administration of a high-dose recombinant Nipah vaccine candidate formulated with SWE adjuvant demonstrated a favorable systemic safety profile in Syrian hamsters. Histopathological alterations

were limited to mild, low-grade cellular changes in the liver, lungs, and kidneys, while the brain, heart, intestine, and gonads showed no treatment-related lesions. Splenic white pulp hyperplasia was consistent with a reactive immune response following formulation exposure. Evaluation under the Patterned Physiological Criteria framework provided preliminary safety metrics supporting host tolerance across clearance tissues. These findings provide preclinical safety data to support continued development of the candidate vaccine formulation.

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NOVELTY STATEMENT

This study provides the first comprehensive multi-organ histopathological baseline safety profile of a high-dose recombinant Nipah virus attachment (G) glycoprotein seed vaccine candidate formulated with a squalene oil-in-water emulsion (SWE) adjuvant in Syrian hamsters. Beyond establishing organ-level tolerance under a worst-case repeated-dose regimen, this work introduces an integrative “Patterned Physiological Criteria” exploratory analytical framework to differentiate expected, co-directional physiological clearance adaptations from uncoupled systemic toxicity across eight visceral organs. Standardized under modern INHAND diagnostic criteria, these findings demonstrate that the SWE-adjuvanted formulation maintains broad systemic safety without inducing irreversible tissue lesions or sex-specific toxicological variations, establishing a foundational preclinical benchmark for future Henipavirus vaccine platforms.

AUTHOR'S CONTRIBUTION

Conceptualization: RVN, SI, and CAN. Methodology: RVN, SI, and CAN. Software: ZIA, ANN, EAR and IYR. Validation: RVN, KPS, KR, DL, JR, and LRY. Formal analysis: ANN, IYR, EAR. Investigation: EDP, TWM, and RG. Resources: RVN, SI, and ANN. Data curation: SI, EDP, and TWM. Writing original draft: ZIA. Writing review and editing: ZIA and EDP. Visualization: ZIA, EDP, TWM, and RG. Supervision: RVN, SI and CAN. Project administration: ZIA, SI, and TWM.

GENERATIVE AI AND AI ASSISTED TECHNOLOGY STATEMENT

The authors declare that generative AI was used solely

for language refinement and grammatical improvement. The authors take full responsibility for the content of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tabel S1: Consolidated table of abbreviations.

Abbrevia- tion	Full Technical Definition
ARDS	Acute Respiratory Distress Syndrome
ATP	Adenosine Triphosphate
BPOM	Badan Pengawas Obat dan Makanan (Indonesian FDA)
BSL-3	Biosafety Level-3
EMA	European Medicines Agency
G-protein	Attachment Glycoprotein
HE	Hematoxylin and Eosin
IM	Intramuscular
INHAND	International Harmonization of Nomenclature and Diagnostic Criteria
Na ⁺ /K ⁺ ATPase	Sodium-Potassium Adenosine Triphosphatase Pump
NBF	Neutral Buffered Formalin
NiV	Nipah Virus
PALS	Periarteriolar Lymphoid Sheath
PMN	Polymorphonuclear Leukocyte
SPF	Specific-Pathogen-Free
SWE	Squalene Oil-in-Water Emulsion
US-FDA	United States Food and Drug Administration
WHO	World Health Organization